Application Note

Effects of low A_{260}/A_{230} ratios in RNA preparations on downstream applications

The efficiency of applications such as real-time RT-PCR and RNA-seq depends strongly on the purity of the RNA sample used. To assess RNA purity, the absorbance of RNA at 260 nm and the absorbance of potential contaminants at 280 nm or 230 nm was determined by spectroscopic measurement (e.g., QIAxpert® or NanoDrop®). An A_{260}/A_{280} ratio of 1.8–2.1 at pH 7.5 is widely accepted as indicative of highly pure RNA. Pure RNA should also yield an A_{260}/A_{230} ratio of around 2 or slightly higher; however, there is no consensus on the acceptable lower limit of this ratio. Also, it has not been fully established which contaminants contribute to a low A_{260}/A_{230} ratio. Possible candidates include salt, carbohydrates, peptides and aromatic compounds such as phenol (1, 2). In our experience, increased absorbance at 230 nm in RNA samples is almost always due to contamination by guanidine thiocyanate, a salt which absorbs very strongly at 220–230 nm and can be present at very high concentrations in the lysis buffer or extraction reagent (e.g., TRIzol®) used in most RNA purification procedures. Our experiments showed that the A_{260}/A_{230} ratio of an RNA sample is strongly reduced when guanidine thiocyanate is present even at submillimolar concentrations (Figure 1A). However, we also found that concentrations of guanidine thiocyanate of up to 100 mM in an RNA sample do not compromise the reliability of real-time RT-PCR, even when using PCR chemistries that are sensitive to inhibitors (Figure 1B). Similar observations have been reported by other researchers (3).

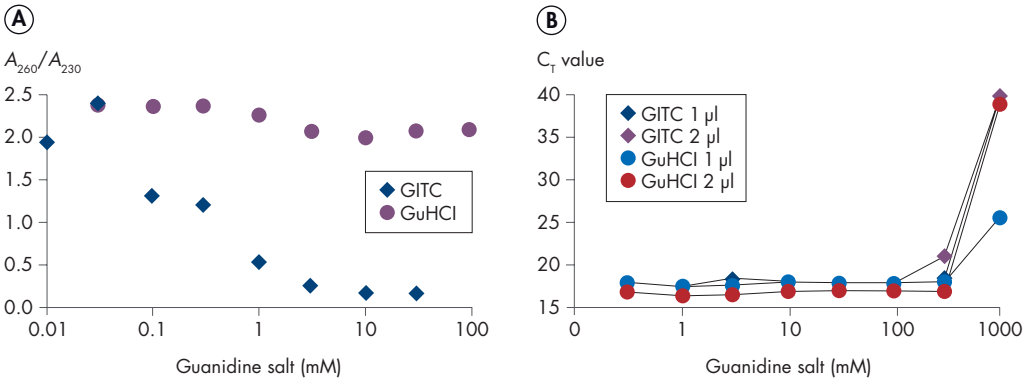


Figure 1. Effect of guanidine salt concentration on the A_{260}/A_{230} ratio and real-time RT-PCR. (A) Ratio of A_{260} to A_{230} for RNA samples (50 ng/µl) containing 0.03–100 mM guanidine hydrochloride (GuHCl) or 0.01–30 mM guanidine thiocyanate (GITC). (B) C_T values obtained from real-time one-step RT-PCR using a TaqMan® Gene Expression Assay for beta-actin and a master mix from Supplier AII. The reaction volume was 25 µl, and the template was either 1 µl or 2 µl of a 50 ng/µl RNA sample containing 0.3–1000 mM guanidine salt.

Impact of RNA concentration and contaminants on the A_{260}/A_{230} ratio

When establishing a suitable lower limit for the A_{260}/A_{230} ratio, it is important to remember that this ratio (and other absorbance ratios in general) also depends on RNA concentration. Trace amounts of contaminants will have virtually no effect on the ratio if the RNA is at a high concentration, but will have a major impact on the ratio if the RNA concentration is low. However, the most important factor is the amount of contaminant that is transferred to the downstream reaction (e.g., cDNA synthesis), rather than the absorbance ratio.

Example with low mass input samples

A low A_{260}/A_{230} ratio can also be influenced by a low starting sample amount in the RNA extraction procedure, leading to a low concentrated RNA sample after extraction. Despite recommendations to use a certain amount of starting material, sometimes the amount is limited due to the source of material or availability (e.g., tissue, biopsies, etc.). Smaller sample amounts can therefore result in lower A_{260}/A_{230} ratios in comparison to recommended starting amounts. The RNA samples below (from 5 or 0.5 mg brain extracted using the miRNeasy Micro Kit either manually or a QIAcube® for automated purification procedure) had a normal A_{260}/A_{280} ratio around 1.8–2.1, but reduced A_{260}/A_{230} ratios especially for the 0.5 mg samples (Table 1). The A_{260}/A_{230} ratios for the low mass input samples were within 0.8–1.5.

		OD 280 nm	OD 260 nm	A_{260}/A_{280}	A_{260}/A_{230}	ng/µl	Vol.	Yield	Mw	Std. Dev.
5 mg	QIAcube	10.653	5.159	2.06	1.81	426.1	13.5	5.752	5.731	0.022
		10.573	5.139	2.06	1.81	422.9	13.5	5.709		
	Manual	10.089	4.889	2.06	1.75	403.6	11.5	4.641	4.702	0.060
		10.354	5.05	2.05	1.95	414.1	11.5	4.762		
0.5 mg	QIAcube	1.036	0.512	2.02	1.51	41.46	14.5	0.601	0.615	0.014
		1.122	0.576	1.95	0.85	44.87	14.0	0.628		
	Manual	1.25	0.625	2	1.29	50.02	11.5	0.575	0.647	0.072
		1.563	0.88	1.78	0.89	62.54	11.5	0.719		

Table 1. Effect of standard and low mass sample input on A_{260}/A_{230} ratio calculated from absorbance measurements made on a Nanodrop® spectrophotometer. RNA from different amounts of brain tissue were extracted using the miRNeasy® Mini Kit either manually or on the QIAcube.

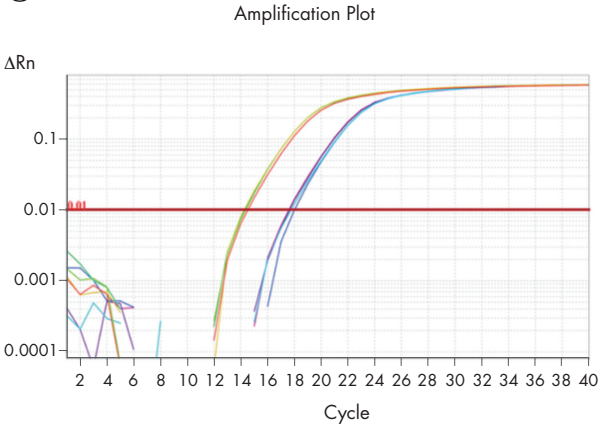
A low A_{260}/A_{230} ratio does not influence downstream applications

Although the A_{260}/A_{230} ratio is low, no influence or inhibition is observed in downstream applications, such as RT-PCR (Figure 2A). In our experiments, the real-time PCR run for the samples with a low A_{260}/A_{230} ratio resulted in typical amplification (Figure 2B) and melting curves (Figure 2C). The ΔC_T for both sample input amounts was approximately 3.3, independent of the extraction procedure. Although this analysis was performed using real-time RT-PCR, similar conclusions apply to microarray analysis and other applications that rely on cDNA synthesis as the first step — the step that is the most influenced by contaminants.

Ⓐ

		C_T sample 1	C_T sample 2	Mw	Std. Dev
5 mg	QIAcube	14.569	14.318	14.444	0.126
	Manual	14.409	14.323	14.366	0.043
0.5 mg	QIAcube	17.769	18.068	17.919	0.150
	Manual	17.697	17.612	17.655	0.043

Ⓑ



Ⓒ

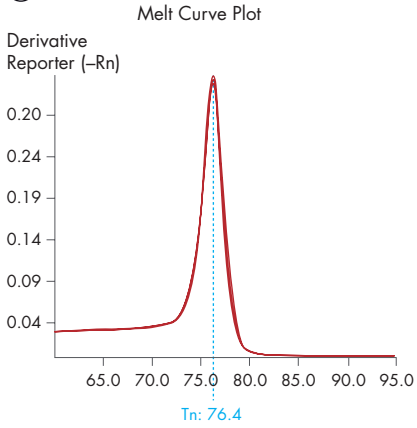


Figure 2. Detection of mir16 in a SYBR® Green RT-PCR Assay. (A) C_T values obtained from real-time one-step RT-PCR using a miScript® Gene Expression Assay for mir16. The same sample template volumes were used for all reactions. (B) Amplification plots and (C) melting curves showed comparable curves and a single melting peak, indicating homogeneous amplification.

QIAxpert® and Spectral Content Profiling

The QIAxpert is an innovative µ-volume UV/Vis spectrophotometer that overcomes limitations of classic spectrophotometry and purity assessment using absorbance ratios.

Using reference spectra of known contaminants, a state of the art software algorithm on the QIAxpert instrument performs a deconvolution of measured UV/Vis spectra according to the Beer Lambert law for mixtures, stating that the absorption spectrum of a mixture is a linear combination of the spectra of its individual constituents. This feature, known as Spectral Content Profiling (SCP), allows dye-free and easy differentiation between DNA, RNA and other UV/Vis absorbing contaminants in complex biological samples.

The QIAxpert enables yield and purity assessment in a single measurement while offering accurate dye-free DNA, RNA fractions quantification irrespective of the buffering conditions and assessment of the amount of contributing quantities of co-purified substances including sample turbidity, contaminating nucleic acids, phenol and other contaminants present in the sample. Our experiments showed that it is possible to get reliable RNA quantification despite increasing guanidine thiocyanate contamination and decreasing A_{260}/A_{230} ratios in 50 ng/µl RNA (Figure 3). However, guanidine thiocyanate in RNA in applied concentrations for QIAxpert measurements had no influence on C_T values obtained with real-time one-step RT-PCR (Figure 1B).

Position	Sample name	RNA (ng/µl)	Nucleic acids (ng/µl)	Impurities (A_{260})	Background (A_{260})	Residue (%)	A_{260}	A_{260}/A_{280}	A_{260}/A_{230}
A1	RNA	49.9	49.9	0.00	1.10	1.10	1.24	1.80	2.34
B1	RNA + 0.3 mM GITC	50.9	50.9	0.00	0.74	0.74	1.28	2.06	0.74
C1	RNA + 0.6 mM GITC	50.7	50.7	0.02	0.96	0.96	1.28	2.03	0.54

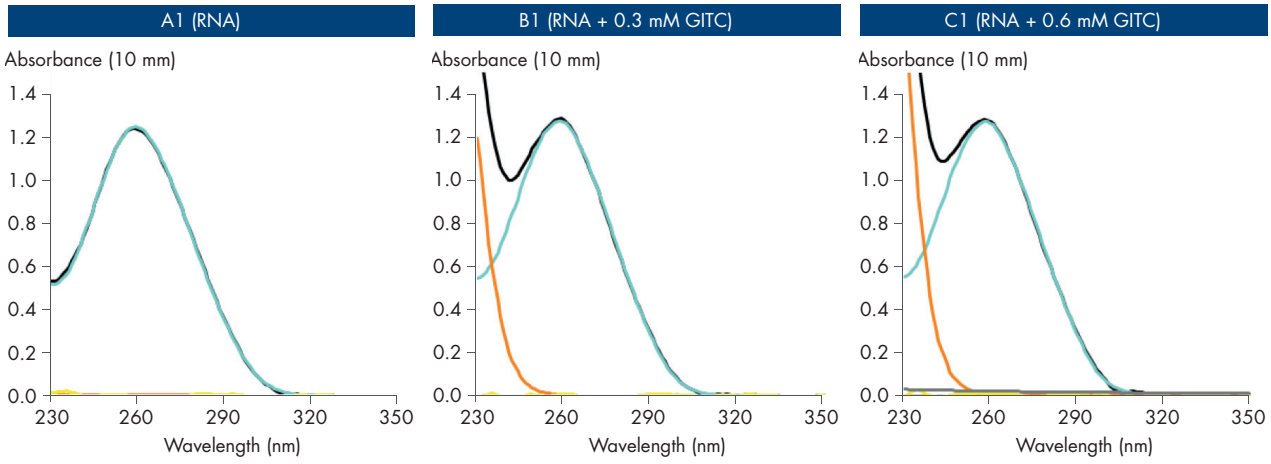


Figure 3. QIAxpert measurements with RNA. RNA concentration and purity were measured on QIAxpert with and without spiked-in guanidine thiocyanate contamination. (Top) Results table and (bottom) spectral content profiling spectra, showing total measured spectrum (black); RNA spectrum (blue); impurities spectrum, including guanidine thiocyanate (orange); residues (yellow); and background (gray). The RNA concentration was the same regardless of increasing guanidine thiocyanate contamination.

Note: Some sources have reported that the absorbance maximum for guanidine thiocyanate is around 260 nm (4), which is similar to that for RNA (Figure 4A). This observation appears to be due to a saturation effect on the Nanodrop spectrophotometer at high concentrations of the salt, about 10–1000 times higher compared to what may be observed in RNA isolated by GTC-based methods (Figure 4A). This would incorrectly lead to an optimal A_{260}/A_{230} ratio and an overestimation of RNA concentration at high GITC contamination on a Nanodrop. At moderate or low salt concentrations, the absorbance maximum is around 220–230 nm, leading to low A_{260}/A_{230} values. On other spectrophotometers, such as the SpectraMax® or the QIAxpert, the right shoulder of the GITC absorption spectrum shifts to the right at higher concentrations, due to saturation, while the absorbance maximum remains below 230 nm wavelength (Figure 4B–C). In conclusion, while QIAxpert can estimate RNA concentrations precisely despite moderate contamination with GITC (Figure 3), at very high GITC concentrations, QIAxpert and SpectraMax would overestimate RNA concentrations with low A_{260}/A_{230} values. The Nanodrop would also overestimate RNA concentration and incorrectly show good A_{260}/A_{230} values.

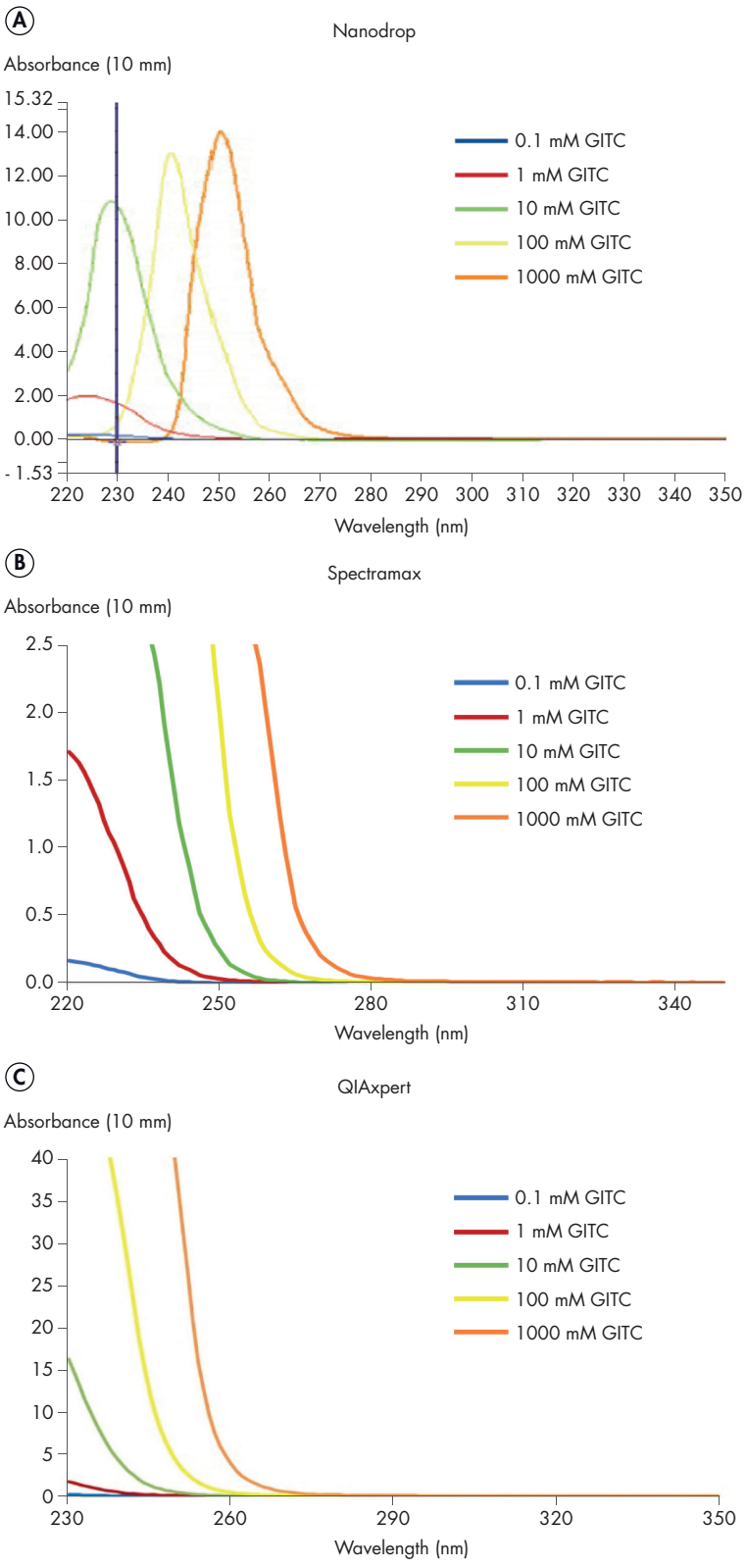


Figure 4. Effect of high guanidine thiocyanate concentrations on absorbance measurements made on (A) Nanodrop spectrophotometer, (B) Spectramax and (C) QIAxpert. The wavelength for peak absorbance appears to increase at high salt concentrations on a Nanodrop, while SpectraMax and QIAxpert showed a shift of the right shoulder due to saturation.

References

1. Teare JM, Islam R, Flanagan R, Gallagher S, Davies MG, Grabau C. (1997) Measurement of nucleic acid concentrations using the DyNA Quant and the GeneQuant. Biotechniques. **6**, 1170. **www.bcm.edu/mcfweb/?PMID=3100**
2. Gallagher SR. Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. (2011) Curr Protoc Mol Biol. 10.1002/0471140864.psa04ks52.
3. Cicinnati, V.R., Shen, Q., Sotiropoulos, G.C., Radtke, A., Gerken, G., and Beckebaum, S. (2008) Validation of putative reference genes for gene expression studies in human hepatocellular carcinoma using real-time quantitative RT-PCR. BMC Cancer. **8**, 350.
4. Thermo Scientific (2010). T042-Technical Bulletin NanoDrop Spectrophotometers, 260/280 and 260/230 Ratios [**online**].

Ordering Information

Product	Contents	Cat. no.
miRNeasy Micro Kit (50)	For 50 total RNA preps: 50 RNeasy MinElute® Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol® Lysis Reagent, RNase-Free Reagents and Buffers	217084
QIAcube (110 V)	Robotic workstation for automated purification of DNA, RNA, or proteins using QIAGEN spin-column kits: includes 1-year warranty on parts and labor	9001292
QIAxpert	QIAxpert instrument with 1 year warranty coverage including parts, labor and shipping	9002340
miRNA16miScript Primer Assay (100)	10x miScript Primer Assay (contains one miRNA-specific primer)	Varies
miScript II RT Kit (12)	For 12 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218160
miScript SYBR Green PCR Kit (200)	For 200 reactions: QuantiTect® SYBR Green PCR Master Mix, miScript Universal Primer	218073

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Visit **www.qiagen.com/geneglobe**.